Does each bead count? A reduced-cost approach for recovering waterborne protozoa from challenge water using immunomagnetic separation

Natália de Melo Nasser Fava, Kamila Jessie Sammarro Silva, William John Snelling, Nigel George Ternan, James Stephen Gerard Dooley and Lyda Patricia Sabogal-Paz

ABSTRACT

Giardia duodenalis and *Cryptosporidium* spp. are two of the most prominent aetiological agents of waterborne diseases. Therefore, efficient and affordable methodologies for identifying and quantifying these parasites in water are increasingly necessary. USEPA Method 1623.1 is a widely used and validated protocol for detecting these parasites in water samples. It consists of a concentration step, followed by parasite purification and visualization by immunofluorescence microscopy. Although efficient, this method has a high cost particularly due to the immunomagnetic separation (IMS) step, which is most needed with complex and highly contaminated samples. Based on this, the present study aimed to determine whether it is possible to maintain the efficiency of Method 1623.1 while reducing the amount of beads per reaction, using as a matrix the challenge water recommended by the World Health Organization. As for *Giardia* cysts, a satisfactory recovery efficiency (RE) was obtained using 50% less IMS beads. This was evaluated both with a commercial cyst suspension (56.1% recovery) and an analytical quality assessment (47.5% recovery). Although RE rates obtained for *Cryptosporidium parvum* did not meet Method 1623.1 criteria in any of the experimental conditions tested, results presented in this paper indicated the relevance of the described adaptations, even in challenge water.

Key words | *Cryptosporidium* spp. oocysts, *Giardia* spp. cysts, low-cost recovery methods, parasitic protozoa, recovery efficiency

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HIGHLIGHTS

- The high cost of current protozoa detection methods limits their widespread use in limited settings.
- Immunomagnetic separation improves detection by cleaning the sample.
- Recovery efficiency is maintained for *Giardia duodenalis* with 50% less beads.
- Organisms adhering to beads after dissociation may impact recovery levels.

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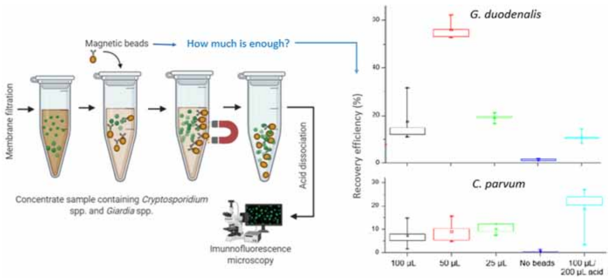
GRAPHICAL ABSTRACT



INTRODUCTION

Some 2.2 billion people around the world do not have safely managed drinking water services, 4.2 billion people are deprived of safely managed sanitation services, and 3 billion lack basic handwashing facilities (WHO 2020). Waterborne diseases are considered one of the highest impact public health problems in the world and are responsible for more than 2.2 million deaths per year and many more cases of enteric infections (WHO 2017).

Almost 40% of these deaths are caused by parasitic protozoa, especially *Giardia duodenalis* and *Cryptosporidium parvum*, which are zoonotic aetiological agents responsible for more than 2.8 million cases per year of gastrointestinal infections worldwide (Squire & Ryan 2017). These infections are the second most common cause of death in early childhood (Checkley *et al.* 2015; Platts-Mills *et al.* 2015).

The repeated prevalence of these protozoa in surface water denotes significant risks to human health, especially due to their low ID50 (the number of cysts and/or oocysts needed to infect 50% of exposed people), which has been estimated to fall between 10 and 2,000 for *C. parvum* (Robert-Gangneux & Dardé 2012) and between 10 and 100 for *G. duodenalis* (Rendtorff 1979). In this context, assessing the microbiological quality of drinking water is mandatory to ensure its safety for consumption (WHO 2017).

Despite the growing trend in pathogen epidemiological investigations in developing countries (Squire & Ryan 2017), the vast majority of studies are still carried out in developed countries, where laboratories and general health infrastructure are much more accessible than those in developing countries (Snelling *et al.* 2007). *Cryptosporidium* is associated with moderate to severe diarrhoea and increased mortality in low-income regions (Sunnotel *et al.* 2006; Snelling *et al.* 2007), and both parasites negatively affect child growth and development (Squire & Ryan 2017). Malnutrition and HIV status are also important contributors to the increased prevalence of *Cryptosporidium* spp. and *G. duodenalis* in developing countries. Climate change and population growth are also predicted to increase both malnutrition and the recurrent prevalence of these parasites in water sources (Squire & Ryan 2017).

Over time, various methodologies have been developed to detect these organisms in water samples. The limitations in early-stage methodologies for protozoan recovery may result in a slight prevalence in surface water, for instance, leading to the incorrect assumption of low contamination (Efstratiou *et al.* 2017a). Also, the efficiency of the critical step, that of oocyst recovery, in these methods is mostly low and extremely variable, ranging from 0 to 140% (Clancy *et al.* 1994; Jakubowski *et al.* 1996; Schaefer 2001). Because of the inconsistency of results, alternative techniques have been proposed and evaluated. Specifically, Method 1623.1, developed by the U.S. Environmental Protection Agency (USEPA), is now recognized as the accepted standard procedure for detecting *Giardia* spp. and *Cryptosporidium* spp. in water. In brief, the method consists of four steps: (1) concentration of the sample (filtration), (2) immunomagnetic separation (IMS), (3) immunofluorescent labelling (IFA), and (4) microscopic visualization of biological forms (USEPA 2012).

Regardless of the detection method employed, large volumes of water are usually required in order to increase the likelihood of detecting cysts and oocysts in the sample. However, the concentration process often leads to an accumulation of debris, such as large organic particles and algal cells, making it necessary to include a sample clarification step, which aims to separate the target organisms from this debris (McCuin *et al.* 2001). In this scenario, IMS is a well-established technique that employs magnetic beads coated with an antibody specific to protein targets on the surface of microorganisms such as *Giardia* spp. and *Cryptosporidium* spp., to allow their recovery from different matrices (Di Giovanni *et al.* 1999; Yakub & Stadterman-Knauer 2000).

Although this technique has operational advantages and presents better results than other methods (Hsu & Huang 2007), the high cost of the immunomagnetic beads severely limits its use in limited-resource situations (Feng *et al.* 2011). Reducing the cost of IMS methodology is, therefore, crucial to ensure that, even in low- and middle-income countries, effective detection of pathogens in water becomes practically feasible. Such a development would lead to standardization of the methodologies across all laboratories and more consistent and reliable results worldwide. As immunomagnetic beads are a primary cost of the method, we, therefore, investigated the efficiency of the IMS method when the number of beads per sample is serially reduced as a step towards achieving this specific goal.

METHODOLOGY

Sampling

Test water consisted in an increase of turbidity and true colour to a natural water source. In short, a 5 L groundwater sample was mixed with humic acid (20 mg L^{-1}) and kaolinite (60 mg L^{-1}) in order to reach about 40 NTU of turbidity, 250 HU of true colour and 10 mg L^{-1} of dissolved organic carbon (DOC). These characteristics represent the so-called challenge water proposed by the World Health Organization (WHO 2014) for water testing.

In our study, 5 L batches were used for each test, and these 5 L batches were divided into five samples of 1 L each.

The groundwater used in this study came from an artesian well which is fed by the waters of the Guarani Aquifer System. The well is located on the campus of the São Carlos School of Engineering, São Carlos, São Paulo, Brazil.

Specifically, for this work, prior to the beginning of the experiments, the well water was submitted to Method 1623.1 (MF + IMS + IFA) for the detection of (oo)cysts of *Giardia* spp. and *Cryptosporidium* spp. The aforementioned method was used for the analysis of all samples included in this study and is, therefore, detailed in the subsection 'Sample processing'.

Protozoa inoculation

Commercially available suspensions of *G. duodenalis* (H3 isolate, 190311) and *C. parvum* (Iowa isolate, 190311; 5×10^6 in 8 mL) (Waterborne, Inc.) were used in order to artificially contaminate the challenge water samples. Viable cysts and oocysts were shipped and stored in phosphate-buffered saline containing antibiotics at 2–8 °C and were utilized within a maximum of 60 days after receipt. Approximately 697 ± 8 cysts and 700 ± 10 oocysts were spiked together into each of four of the 1 L samples, with the remaining 1-L sample being used as a blank control (i.e., without protozoa).

Prior to the tests, the suspensions were analysed to quantify the inoculum. For that, 5 μL of each suspension was spiked on a glass slide, in triplicate, and left at room temperature for 4 h for drying. Next, the commercial kit Merifluor™ (Meridian Diagnosis) and DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride) dye (USEPA 2012) were applied to the sample. Visualization was performed by immunofluorescence microscopy (Olympus® BX51). The final concentration (microorganisms/μL) was given by the average of the results observed in the three slides.

Sample processing

Samples were processed using Method 1623.1 (USEPA 2012) with appropriate adaptations (Medeiros & Daniel 2015; Franco *et al.* 2016; Sammarro Silva & Sabogal-Paz 2020) as described below.

Sample concentration

Vacuum pump filtration (flow rate 4 L/min) using cellulose ester membranes (47 mm diameter, 3 μm porosity, Millipore™) was performed for concentrating the target organisms from 1 L samples (Franco *et al.* 2012).

After the filtration process, the material retained in the membrane was eluted by washing the membrane with 0.01% Tween 80 solution at 45 °C and scraped out using plastic handles supplied with the Merifluor™ kit. Membrane scrapings were carried out for 3 min, in each of the directions (vertical, horizontal and diagonal) covering the entire area of the membrane (Medeiros & Daniel 2015; Sammarro Silva & Sabogal-Paz 2020).

The resulting liquid was then subjected to a double centrifugation process (1,500 × *g*; 10 min; room temperature) to form a pellet containing the target parasites. At the end of the process, samples were resuspended in 5 mL of appropriate kit buffer, and then subjected to IMS in order to purify the protozoa.

It is worth mentioning that throughout the filtration technique, the filter membranes may need to be replaced if they clog and interrupt the flow of the sample. The number of membranes used depends directly on the characteristics of the study water.

Sample purification and protozoa isolation

The Dynabeads™ GC-Combo (Applied Biosystem) kit was applied in this step following the manufacturer's recommendations; this kit was also used for the dissociation step, which was carried out three times using 100 μL of 10% hydrochloric acid, in each time. As part of our aim to obtain an effective but more affordable methodology, assays were performed under four different conditions with a serial reduction in the number of beads in each. The first assay was performed according to the standard protocol of Method 1623.1, in which 100 μL of each bead type was added to the sample. For the second assay, the bead volume was reduced by 50%, and, in the third, the final amount of bead added to the sample was 25% of the standard protocol. The 4th assay was performed without any beads. Apart from these reductions, all other conditions were kept the same for each assay.

Considering the results obtained during the tests, an extra test was included in order to investigate whether the addition of double the volume of 10% hydrochloric acid (200 μL) in the standard amount of beads (100 μL) would positively influence RE.

Microorganism visualization

At the end of each of the three rounds of acid dissociation, 50 μL of the sample (non-adhered material) was recovered and added directly to one of the wells of the glass slide supplied with the Merifluor™ Kit, which was previously sensitized with 5 μL of sodium hydroxide 1 M.

After the drying period of the samples on slides (4 h), (oo)cysts were stained using the commercial Merifluor™ (Meridian Diagnosis) kit and visualized by immunofluorescence light microscopy (Olympus® BX51). As a confirmatory test, DAPI dye was added to all the samples (USEPA 2012).

Recovery rate

Recovery efficiency (RE) of the method is calculated according to Equation (1), where RE is the recovery rate after the complete protocol (%); C_1 is the (oo)cysts enumerated in the first acid dissociation; C_2 is the (oo)cysts enumerated in the second acid dissociation; C_3 is the (oo)cysts enumerated in the third acid dissociation; and NP is the number of inoculated protozoa.

$$\mathrm{RE} = \frac{(C_1 + C_2 + C_3)}{\mathrm{NP}} \times 100\% \tag{1}$$

Analytical quality assay

In order to ensure the reliability of the results obtained in this work, a test with ColorSeed™ was performed, according to Method 1623.1 (USEPA 2012). Colorseed™ reagent contains between 98 and 102 inactivated and permanently red-labelled *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts (with a standard deviation of 2.5 or less) in 1 mL of saline solution. This examination was performed with 50% of the standard bead volume, which was statistically determined as the best option, as outlined in the 'Results' section. Briefly, 2 mL of 0.05% (vol/vol) of Tween 20® was added to the ColorSeed™ tube, which was vortexed and added to 1 L of challenge water, following which the sample containing the cysts and oocysts was subjected to membrane filtration, the selected IMS protocol, and immunofluorescence for microscopic visualization. Likewise, to ensure the safety of results the Colorseed™ test with 50 μL of each bead was also performed in quadruplicate plus blank test, and the RE was determined using Equation (1).

Statistical analyses

Statistical analyses that led to an understanding of differences obtained by each immunomagnetic procedure were performed using PAST 3.2 software (Hammer *et al.* 2001), and Origin 6.0 was used for plotting results. A Shapiro–Wilk Normality test was conducted to determine if the datasets were normally distributed. Both the analysis of variance (ANOVA) and the non-parametric test of Kruskal–Wallis followed by Tukey's pairwise and Dunn's *post hoc*, respectively, were performed. Significantly different results ($\alpha = 0.05$) provided conditions to the analytical quality analysis.

RESULTS

Recovery efficiency

The cysts of *G. duodenalis* and oocysts of *C. parvum* were clearly observed against the background in all samples (Figure 1), regardless of the condition of the test, following the first and second acid dissociations. After the third round of acid dissociation, no cysts or oocysts were visualized. RE data obtained from different methodologies carried out in this study are compiled in Table 1.

As for operational purposes, it is worth mentioning that five membranes were used in each batch filtration.

Figure 2 displays the obtained results for each recovery assay, considering both (a) *G. duodenalis* and (b) *C. parvum* as target organisms against Method 1623.1 minimum RE requirement for each parasite. With *G. duodenalis*, all of the tests, with the exception of the no-bead run, successfully reached the USEPA Method 1623.1 RE recommended range (8–100%). With *C. parvum*, however, none of the USEPA Method 1632.1 tests was satisfactory, as all results were below 32%.

However, when analysing the coefficient of variation (CV) of each test, only one test (100 μL of beads) was not in accordance with the USEPA criteria for *Giardia* spp. The scenario was the opposite concerning *C. parvum*, for which only one condition (25 μL of beads) reached valid values (CV = 20%).

Statistical analysis

The Shapiro–Wilk test indicated that data for percent *C. parvum* recovery without adding beads did not follow a normal distribution. Although the boxplot shown in Figure 2 perhaps visually suggests that the data is normally distributed, this hypothesis was not confirmed. Therefore, *C. parvum* recoveries were analysed by non-parametric statistics. Although the Kruskal–Wallis test suggested significant

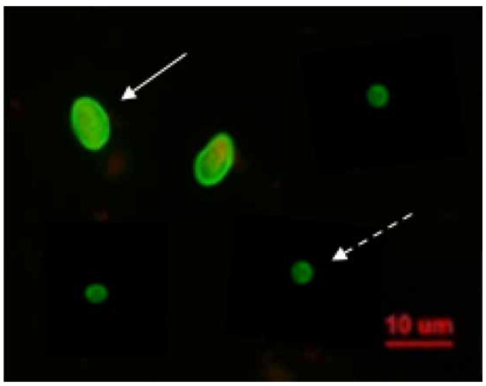


Figure 1 | *G. duodenalis* cysts (white arrows) and *C. parvum* oocysts (dashed white arrows) following membrane filtration and IMS purification and stained with Merifluor kit (FITC) in an immunofluorescence light microscopy (Olympus® BX51) under a 40X objective.

Table 1 | Recovery efficiencies for *G. duodenalis* cysts and *C. parvum* oocysts recovered from spiked challenge water samples using different volumes of immunomagnetic beads (quadruplicate trials plus blank test)

	G. duodenalis		*C. parvum*	
Experimental condition	**RE (%)**	**CV (%)**	**RE (%)**	**CV (%)**
100 μL beads	17.4	48	7.2	70
50 μL beads	56.1	7	9.1	49
25 μL beads	19.1	9	10.3	20
No beads	1.4	21	0.7	64
100 μL beads/200 μL acid	11.0	20	18.8	49
Method 1623.1 USEPA	8–100%	≤39	32–100%	≤37

Notes: Average *G. duodenalis* inoculum: 697 ± 8 cysts; Average *C. parvum* inoculum: 700 ± 10 oocysts; RE, recovery efficiency; CV, coefficient of variation. The negative control tests did not display any autochthone protozoa. RE was calculated using Equation (1).

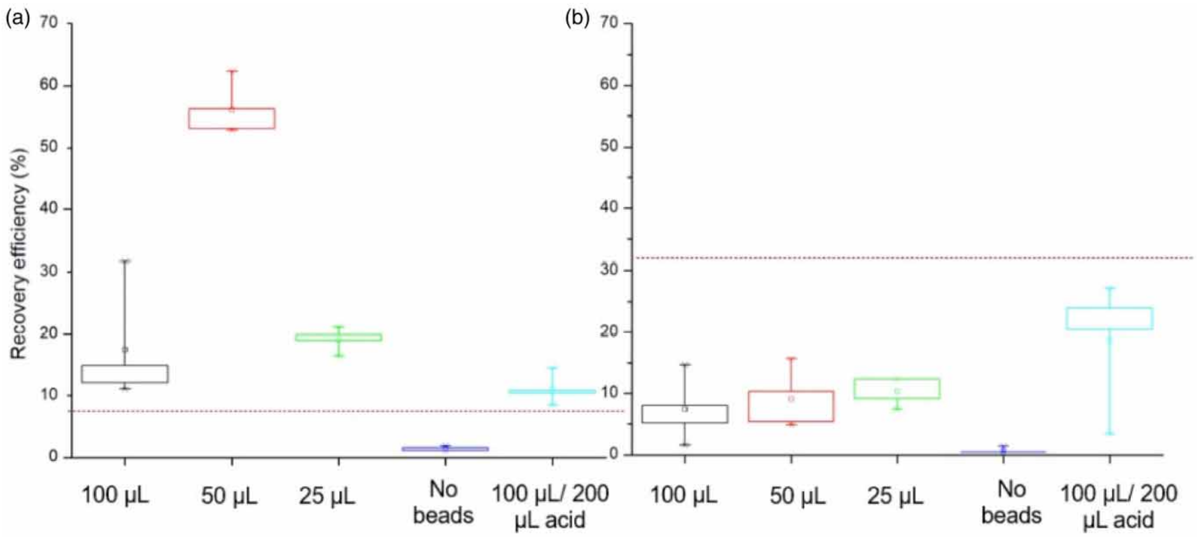


Figure 2 | Recovery efficiencies for different experimental conditions displayed in boxplots for (a) *G. duodenalis* and (b) *C. parvum*. Horizontal dashed lines indicate the USEPA (2012) minimum required recovery values for each parasite. Volumes (µL) refer to the amount of immunomagnetic beads added to each test, as well as the extra procedure considering a different volume in 10% hydrochloric acid during dissociation.

differences among medians of the *C. parvum* datasets and, Dunn's *post hoc* ($p < 0.05$) pointed out that this fact was mainly due to the combinations of beads versus no beads (50, 25, and 100 µL with 200 µL acid, specifically) as significantly different ($p < 0.05$). Considering, in addition, that these results did not meet USEPA (2012) criteria, as illustrated by the dashed line in Figure 2(b), *G. duodenalis* recoveries were prioritized for the analytical quality assessment. A comparison among all experimental conditions is shown in Table 2 which shows that the 50 µL-bead dosing led to significant differences in the sample means against all of the other conditions.

Table 2 | Statistical comparison of the experimental conditions for *Giardia* cyst recovery in challenge water (Tukey's test for 95% confidence interval)

Recovery methods compared	*p*-value
100 µL versus 50 µL	**<0.0001**
100 µL versus 25 µL	0.9889
100 µL versus no beads	**0.0029**
100 µL versus 100 µL beads/200 µL acid	0.3927
50 µL versus 25 µL	**<0.0001**
50 µL versus no beads	**<0.0001**
50 µL versus 100 µL beads/200 µL acid	**<0.0001**
25 µL versus no beads	**0.0011**
25 µL versus 100 µL beads/200 µL acid	0.1976
No beads versus 100 µL beads/200 µL acid	0.0953

Note: bold values indicate significant differences in means.

Analytical quality assessment

Colorseed™ was used to validate the lowest IMS bead concentration that still provided an acceptable RE value. This was determined to be 50 µL of each bead suspension dissociated with two rounds of 100 µL of 10% hydrochloric acid. Under these conditions, RE reached similar values to those of a test with commercial protozoan suspensions (Table 1). Comparing the values obtained herein with those standardized by Method 1623.1, our data were satisfactory for *Giardia* spp. regarding both RE (47.5%) and CV (4%). Concerning *Cryptosporidium* spp., RE was 17% below the value recommended by Method 1623.1, while the CV for *Cryptosporidium* spp. met the USEPA criteria (7.1%).

Cysts and oocysts attached to the beads

In order to verify the efficiency of the acid dissociation procedure, 50 µL of the bead suspensions obtained at the end of the IMS step were stained with Merifluor™ and imaged using fluorescence microscopy. Figure 3 displays the image captures of the best experimental condition obtained in

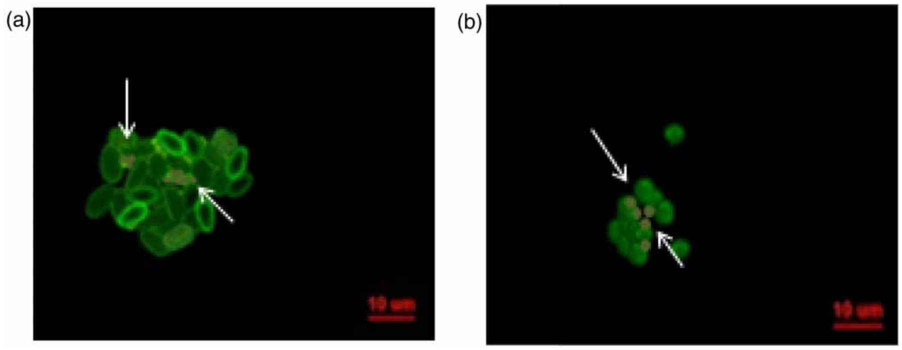


Figure 3 | Cysts of *G. duodenalis* (a) and oocysts of *C. parvum* (b) attached to the immunomagnetic beads following three dissociation procedures, stained with Merifluor kit (FITC) and visualized under a 40× objective. Arrows point to the beads location.

this study (50 μL of each bead and 100 μL of 10% hydrochloric acid). Visual analysis revealed that cysts and oocysts were still attached to the magnetic beads.

It was determined that, on average, 40 and 31% of the total inoculated cysts of *G. duodenalis* (697 ± 8) and oocysts of *C. parvum* (700 ± 10), respectively, remained adhered to the beads. These values are calculated according to Equation (2), where PA is the total amount of protozoan adhered to the beads after three acid dissociation procedures (%); P_1 is the (oo)cysts attached to the beads after the first acid dissociation; P_2 is the (oo)cysts attached to the beads after the second acid dissociation; P_3 is the (oo)cysts attached to the beads after the third acid dissociation; and NP is the number of inoculated protozoa.

$$\mathrm{PA} = \frac{(P_1 + P_2 + P_3)}{\mathrm{NP}} \times 100\% \tag{2}$$

DISCUSSION

The quality of water resources is a fundamental aspect of the public water supply. Although USEPA Method 1623.1 is widely used and is reported in approximately 30% of publications regarding monitoring of *Cryptosporidium* spp. and *Giardia* spp. in water (Efstratiou *et al.* 2017b), it still presents some limitations mainly related to its high cost. Among the steps of the method, IMS represents the highest cost, especially because there is only one supplier of magnetic beads and buffers for both protozoa (Dynabeads® GC-Combo, Life Technologies, Thermo Fisher Scientific Inc.), and therefore, the option of using alternative beads is excluded.

In the current work, RE results ranged from 0.7 to 56.1% for both protozoa (Table 1). These findings are in accordance with the literature that reported equated values to the aforementioned method for *Giardia* spp. and *Cryptosporidium* spp. (Razzolini *et al.* 2010, 2011; Stancari & Correa 2010; Feng *et al.* 2011; Ongerth 2013; Sato *et al.* 2013; Franco *et al.* 2016; Maciel & Sabogal-Paz 2016; Pinto *et al.* 2016). The coefficients of variation obtained (Table 1) reaffirm the high variability inherent to research with protozoa (Francy *et al.* 2004). Similar limitations have been reported by studies on (oo)cyst recovery with and without purification methods (Lora-Suarez *et al.* 2016; Maciel & Sabogal-Paz 2016; Silva & Sabogal-Paz 2021). Hence, this endorses the need for revising recovery protocols.

As indicated by the results obtained herein, in terms of RE, all tests using IMS complied with the criteria of Method 1623.1 for *G. duodenalis* but not for *C. parvum*. One of the experimental conditions, however, must be highlighted: the test performed with 50 μL of each bead solution and 100 μL of 10% hydrochloric acid yielded the highest RE for *G. duodenalis* which agrees with the values established by Method 1623.1. This condition was also statistically significant compared with the others ($p < 0.05$) and was validated by the analytical quality assessment.

Some cysts and oocysts were detected in the absence of IMS, but the RE was insignificant (Table 1).

The fact pointed out by the results of this study that the IMS methodology regardless of the number of beads was efficient only for the recovery of G. *duodenalis* cysts was also reported by other researchers such as Stancari & Correa (2010), Ongerth (2013), Franco *et al.* (2016), and Maciel & Sabogal-Paz (2016). These studies did not obtain satisfactory results for *Cryptosporidium* spp. recovery either.

Data obtained in our research also endorse the importance of two rounds of acid dissociation from the beads, corroborating the findings of Maciel & Sabogal-Paz (2016). Our protozoa recovery from challenge water also suggests that the third dissociation may be dismissed since no organisms were visualized after it, further reducing the cost of the protocol, as no labelling of an extra microscopy slide should be required for immunofluorescence.

Our revised methodology represents a significant improvement compared with those previously carried out. Particularities inherent to the matrix and the methodology itself may influence results. The WHO challenge water, which was used in this study, presents a much higher turbidity than filtered or treated water from water treatment plants (WTPs). Hence, it contains a greater amount of suspended particles, which directly impact upon this methodology performance (Kothavade 2012; Efstratiou *et al.* 2017a, 2017b). When there are too many solids or colloidal material in the sample, the elution process is hampered as the particles remain trapped within the membrane. This may lead to a decrease in the ratio of recovered cysts and oocysts (Franco *et al.* 2012), which would also explain the low recovery rates of *C. parvum* in the present study. Also, the high turbidity of the samples resulted in a greater number of membranes being used in the protozoa protocol, since they were quickly obstructed by the particles present in the water sample. The five membrane replacements per litre of challenge water may have facilitated the dispersion and loss of parasites, as pointed out by Franco *et al.* (2012) and Maciel & Sabogal-Paz (2016).

Although the loss of cysts and oocysts is observed throughout the process (Kumar *et al.* 2016; Pinto *et al.* 2016), the filtration step itself seems to have a great impact on the results. Feng *et al.* (2003) and Hu *et al.* (2004) endorse this statement reporting 92.0 and 89.0% of RE for *Cryptosporidium* spp. oocysts and *Giardia* spp. cysts, respectively, when the water sample is *not* filtered. However, when the filtration step is incorporated into the methodology, these authors reported that the RE declined to 18.1% for *Cryptosporidium* spp. and 77.2% for *Giardia* spp.

The slight decrease observed in the RE for *G. duodenalis* cysts, which consequently makes it more representative than the RE for *C. parvum* – as per the results of the present study – can be attributed to the size of the organisms (Hsu & Huang 2000; Hashimoto *et al.* 2002; Hu *et al.* 2004; Franco *et al.* 2016). The cysts of *G. duodenalis* (8–12 μm) are significantly larger than the oocysts of *Cryptosporidium* (4–6 μm) (USEPA 2012) and, therefore, are more easily retained by the membrane. In addition, *Cryptosporidium* spp. oocysts have the ability to compress (Li *et al.* 1995), which may facilitate their passage through the filter matrix, therefore also contributing to lower recovery.

Although some authors recommend the use of membranes with smaller porosity in order to retain more (oo)cysts, the 3 μm porosity membrane has been extensively used, with favourable results (Franco *et al.* 2012, 2016; Medeiros & Daniel 2015; Pineda *et al.* 2020; Sammarro & Sabogal 2020). Additionally, as reported by Franco *et al.* (2012), the filtration using this kind of membrane presents a better performance in face of complex matrices, such as the one included in this study, than the filters with smaller porosities. It also redeems a generally lower cost as it would require even fewer replacements, which reinforces the main idea of this work, which refers to savings in material – while maintaining response reliability – in order to make the methodology more accessible and widespread.

Another variable worth pointing out in the context of non-satisfactory RE is the continued attachment of cysts and oocysts to immunomagnetic microspheres, even after two rounds of acid dissociation. Similar observations were made by Rochelle *et al.* (1999), Maciel & Sabogal-Paz (2016), Pinto *et al.* (2016), Andreoli & Sabogal-Paz (2019), and Ogura & Sabogal-Paz (2021). This suggests that the acid dissociation step proposed by Method 1623.1 is not fully efficient. For *G. duodenalis* cysts, although the most effective condition obtained in our study used twice as much acid in relation to the amount of each bead, this alone does not seem to be a determining factor for improving the dissociation process, since by maintaining this proportion but using 100 μL of each bead and 200 μL of acid, the results were not satisfactorily proportionate.

Although the best result of this work was only for one target microorganism (i.e., *G. duodenalis*), the achieved results represent a significant improvement regarding the cost–benefit of the protozoa detection protocol. The expense for processing a single water sample is approximately US \$180 for all the consumables required by Method 1623.1 and considering only the Merifluor™ and Dynabeads™ kits, the cost is estimated at US \$130 per sample, in which 75% of this value is due to the use of the Dynabeads™ kit (Brazilian quote in January 2020). Based on this, the expense for a single protozoan test was over US \$118, significantly higher when compared with the costs of other routine assays required to monitor a water supply system.

These high costs are a limiting factor, especially in low- and middle-income countries, which usually lack the infrastructure, qualified labour and economic resources. This situation can be verified by evaluating publications on protozoa around the world. Almost 70% of the publications using Method 1623.1 are concentrated in Europe and North America, while Africa and Central/South America have only 5% (Efstratiou *et al*. 2017b). According to Giglio & Sabogal-Paz (2018), detecting protozoa in complex matrices is expensive and limits surveillance and control programs in developing countries; thus, more research is needed to make parasite detection possible in these countries and a reduced-cost approach might assist in reaching this goal.

As previously mentioned, the high cost of the methodology does not fall exclusively onto the IMS, but it is, in fact, the main expense. The Merifluor™ kit, additional use of DAPI, the epifluorescence microscope and all the necessary infrastructure to carry out the method are direct contributors to its enhancement. However, none of the aforementioned items/reagents can be removed from the global protocol without causing its mischaracterization and most likely loss of results. In this sense, we opted for the careful optimization of one of the methodological steps as an attempt to generate financial savings. The cost reduction in the IMS procedure is reflected by the increased durability of the kit, which, according to our results, can be used in 100 samples instead of 50, as recommended by the manufacturer. Therefore, the alternative offered by our study (50 μL of beads), which complies with the USEPA criteria at least for *G. duodenalis* allows doubling the capacity of the Dynabeads™ kit leading to a significant reduction in costs. In addition, the inference that the third acid dissociation step is not necessary for the success of the methodology also impacts its cost, as less IFA reagents, DAPI and hydrochloric acid will be required per sample.

CONCLUSIONS

Based on the results obtained from this study, we suggest an adaptation to the purification step described in Method 1623.1 in order to provide a methodology with a better cost–benefit that still provides the recovery rate necessary for (oo)cysts, even from complex matrices.

Although none of the conditions explored here was satisfactory for *C. parvum* oocyst recovery, the results point to a significant cost reduction of *G. duodenalis* cyst detection, since half of the volume of immunomagnetic beads (50 μL) used in our study complied with the USEPA recovery efficiencies.

The development of cost-effective protocols to detect and monitor waterborne parasites in water (e.g., *Cryptosporidium* spp. and *G. duodenalis*) is crucial to more effectively evaluate the water quality in developing countries having a direct impact on public health. However, this will continue to be extremely challenging, not least because scientists in developing countries face lower absolute levels of funding and must often pay far too expensive and unsustainable costs for consumables and equipment.

Further studies are recommended to improve the organism-bead dissociation process, seeking to increase the protozoa detection protocol performance in the sample purification phase.

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CONFLICTS OF INTEREST

The authors hereby declare previous originality check, no conflict of interest and open access to the repository of data used in this paper for scientific purposes.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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